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# Rapid microtiter plate assay for determination of metformin in plasma

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**ABSTRACT**

A rapid, sensitive, and reproducible microtiter plate assay for the determination of Metformin in plasma and water was developed. Plasma or water samples (100 µl aliquots) were prepared by the addition of acetonitrile (1 ml) and then briefly vortex-mixed and centrifuged at 12000 rpm for 20 minutes. Supernatant was taken in a fresh eppendorf tubes and to this 1 ml of dichloromethane was added. Tubes were vortexed and centrifuged at 12000 rpm for 20 minutes. Supernatant was collected and an aliquot 50 µl of each sample was transferred to a 384-well microtiter plate and read spectrophotometrically at 246 nm. Calibration curves were linear over the concentration range of 0.5–4 mg/ml or 0.025–0.4 mg/ml for plasma and water, respectively; correlation coefficients ($r^2$) were >0.9969. The intra and inter-day coefficients of variation in plasma, water were <15%. This method is well suited for the rapid analysis of large numbers of samples and is currently being used for in vitro investigations of metformin.

**Key words:** Metformin, Microtiter plate assay, Plasma.

## INTRODUCTION

Metformin hydrochloride is an oral biguanidine, which reduces the elevated blood glucose concentration in patients with diabetes but does not increase insulin secretion. It does not lower the blood glucose in nondiabetic subjects[1]. Augmentation of muscular glucose uptake and utilization, and reduction of increased hepatic glucose production through an antigluconergic action explain the blood glucose lowering effect[2,3].

Metformin is safe[4] and not teratogenic[5] in many of the species studied. Oral bioavailability of metformin is about 50 - 60% and fecal recovery is about 30%[6]. The rate of absorption was slower than that of elimination, which resulted in a plasma concentration profile of "flip-flop" type for oral metformin[7]. Many HPLC methods for the analysis of metformin in plasma are reported. But most of the methods use either ion pair reagent or cation exchange column[8]. Some methods reported require elaborate sample preparation[9]. Though, these methods are sensitive and reproducible, Elisa method for the estimation of metformin in human plasma are found to be more suitable.

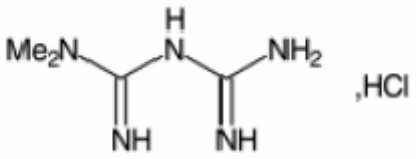
## EXPERIMENTAL

### Materials

Metformin was obtained as gift sample from Microlabs, Bangalore, India. Methanol and acetonitrile of HPLC Grade and potassium dihydrogen orthophosphate, glacial acetic acid, ammonium hydroxide and perchloric acid of GR Grade were purchased from E. Merck (India) Ltd., Mumbai.

### Equipment

In vitro dissolution study was performed using USP 24 Type II dissolution test apparatus (Electrolab TDT-06P, India). The optical density of all samples was measured utilizing a microtiter plate reader (BIO-Tech µQuant, USA) set at 246 nm.




*Metformin hydrochloride*

**Preparation of stock solutions and spiked standards**

Accurately weighed, 400 mg of the metformin was dissolved in 100ml of water to result in a concentration of 4000 µg/ml. Working standards were prepared using this stock solution. Known volume of the stock was diluted with water, to obtain a working standard with the different concentrations of the drug ranging from 400µg/ml to 2000 µg/ml. From these solutions 50 µl each was transferred into eppendorf tube. Different concentrations of drug ranging from 2 to 10 µg/ml were prepared by adding 450 µl of plasma into each tube and vortexed. To each of the tube 1 ml of acetonitrile was added, vortexed and centrifuged at 12000 rpm for 20 minutes. Supernatant was taken in a fresh eppendorf tubes and to this 1 ml of dichloromethane was added. Tubes were vortexed and centrifuged at 12000 rpm for 20 minute. Supernatant was collected and 50 µl was added to each well. The absorption maxima (λ-max) for Metformin in plasma were determined by scanning the drug solution with in the range of 200 to 400 nanometers using a ELISA Spectrophotometer, µQuant. It was found that the drug exhibited a λ-max at 246 nanometers. The absorbance values thus obtained were plotted against the respective concentration to obtain the calibration graph. An average of six determinations was considered to obtain statistically significant results.

**Linearity**

Calibration curves were constructed using ten standard concentrations of Metformin in plasma and water was run in six times. Curves were obtained daily for 3 days. Standard concentration ranges in plasma and water were 0.5 - 5.0 mcg/ml Metformin, where equation was found to be y=0.8689x and $R^2$=0.9969. Individual standard concentrations in plasma and water are shown in Figure 1 and Tables 1-2, respectively.

**Precision and accuracy**

The precision and accuracy of the assay were determined based on analysis of plasma and water QC samples. QC sample concentrations for Metformin were 0.75, 2.0, and 3.5 mg/ml in plasma and water. Six replicate QC samples at each concentration were analyzed on 2 consecutive days, followed by analysis of 12 replicate QC samples at each concentration on the third day, after which intra and inter-day means, standard deviations (S.D.), and relative standard deviations (R.S.D.) were calculated. QC samples were subjected to three freeze-thaw cycles (-80°C to room temperature) to evaluate Metformin stability.

## RESULTS

The method we report demonstrates the successful adaptation of similar visual method to a microtiter plate format, which substantially improves sample throughput. Calibration curves generated using linear least squares regression were linear over the concentration ranges evaluated in plasma and water with all correlation coefficients ($r^2$) >0.9969. The intra and inter-day %R.S.D. in water (Table 1) and plasma (Table 2) ranged from 17.6 to 1.5%. The intra and inter-day% bias ranged from 9.5 to -12.1%. Metformin samples were stable through three freeze-thaw cycles.

Solution stability of Metformin was studied by leaving the solution in tightly capped ambered colour volumetric flasks at room temperature for three days. Content of Metformin was checked for 12 hours interval and compared with freshly prepared solutions. No variation was observed in the content of Curcumin for the study period, which indicates that the Curcumin sample solutions prepared in the said diluents are stable for at least 3 days.

The ruggedness was established by carrying out the assay of Metformin using the same chromatographic system and the same column by two analysts on a different day. The assay results

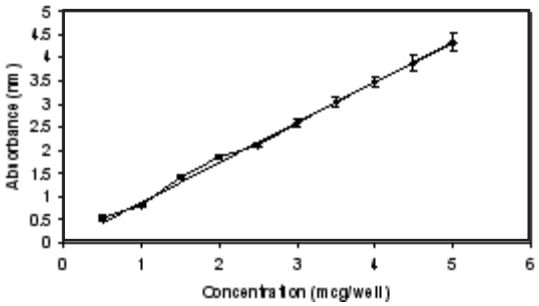



**Fig. 1: Standard Graph of metformin in Water**

**Table 1: Intra- and inter-day precision and accuracy in water**

| | Concentration (mcg/ml) Added | Observed (mean ± S.D.) | %R.S.D. | %Bias |
|---|---|---|---|---|
| [a]Intra-assay | 0.75 | 0.72 ± 0.03 | 4.0 | -4.6 |
| reproducibility | 2 | 2.04 ± 0.03 | 1.7 | 2.0 |
| Quality controls | 3.5 | 3.55 ± 0.09 | 2.4 | 1.5 |
| [b]Inter-assay | 0.75 | 0.72 ± 0.03 | 4.0 | -4.1 |
| reproducibility | 2 | 2.10 ± 0.08 | 3.9 | 4.9 |
| Quality controls | 3.5 | 3.60 ± 0.12 | 3.2 | 2.7 |
| Standards | 0.5 | 0.532 ± 0.01 | 4.2 | 9.5 |
| | 1.0 | 1.422 ± 0.50 | 1.9 | 8.2 |
| | 1.5 | 1.856 ± 0.03 | 3.2 | 2.7 |
| | 2.0 | 2.109 ± 0.05 | 2.6 | 9.5 |
| | 2.5 | 2.109 ± 0.05 | 4.9 | 1.9 |
| | 3.0 | 2.596 ± 0.10 | 3.8 | -2.9 |
| | 3.5 | 3.024 ± 0.09 | 1.8 | -3.6 |
| | 4.0 | 3.467 ± 0.12 | 2.2 | -4.9 |
| | 4.5 | 3.894 ± 0.15 | 1.6 | -2.9 |
| | 5.0 | 4.322 ± 0.18 | 1.2 | -11.5 |

a Six to twelve QC samples per concentration.

b Six to twelve QC samples or two standards per day per concentration for 3 days.

**Table 2: Intra- and inter-day precision and accuracy in plasma**

| | Concentration (mcg/ml) Added | Observed (mean ± S.D.) | %R.S.D. | %Bias |
|---|---|---|---|---|
| [a]Intra-assay | 0.75 | 0.78 ± 0.04 | 4.8 | 3.4 |
| reproducibility | 2 | 2.09 ± 0.04 | 1.9 | 4.5 |
| Quality controls | 3.5 | 3.56 ± 0.08 | 2.4 | 1.6 |
| [b]Inter-assay | 0.75 | 0.76 ± 0.04 | 4.7 | 1.4 |
| reproducibility | 2 | 2.13 ± 0.07 | 3.3 | 6.3 |
| Quality controls | 3.5 | 3.58 ± 0.10 | 2.7 | 2.4 |
| Standards | 2.0 | 1.059 ± 0.02 | 5.2 | -1.6 |
| | 4.0 | 1.941 ± 0.05 | 4.3 | -3.0 |
| | 6.0 | 3.048 ± 0.02 | 4.7 | -4.8 |
| | 8.0 | 3.839 ± 0.05 | 1.5 | -4.2 |
| | 10.0 | 5.074 ± 0.05 | 2.8 | -7.5 |
| | | | | -12.1 |

a Six to twelve QC samples per concentration.

b Six to twelve QC samples or two standards per day per concentration for 3 days.

were found within the acceptance criteria of 93 to 103% w/w, hence the proposed method was said to be rugged.

It is the measure of capacity of an assay to remain unaffected by small but deliberate variations in method parameters and provide an indication of its reliability in normal usage. For the robustness study small variations in solution, detection wavelength and sample quantity have been performed and percentage assay of metformin was calculated. The percentage assay in all varied chromatographic conditions was found within the acceptance criteria.

Most of the methods for the determination of Metformin reported during the last 50 years are time and labor intensive. Although several accurate methods for the determination of Metformin have been reported, their utility for measuring Metformin clinically in large numbers of samples is questionable due to their time and labor intensive nature. Our method is well suited for the analysis of large numbers of samples. Numerous samples are processed and subsequently measured in 384-well microtiter plates simultaneously. The benefits of using 384-well plates have been described previously[10]. Up to 384 plasma and/or water samples (including standards and QCs) are processed and measured in fewer than 2 h with our method.

## DISCUSSION

In conclusion, the method provided excellent sensitivity, accuracy and precision, described here is a rapid, sensitive, and reproducible assay for the determination of Metformin in plasma and water with significant advantages over previously described methods.

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